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The effect of the administration of three different antimicrobial premix formulations via the liquid feeding system on the occurrence of Enterobacteriaceae resistant to tetracycline in the liquid feed for pigs

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Abstract

The oral group treatment is still a common procedure in swine production. This project studied the effect of the application of 3 different formulations of antimicrobial premixes (1. chlortetracycline, 2. chlortetracycline + sulfadimidine + tylosin, 3. sulfadimidine + sulfathiazole + trimethoprim) via the liquid feeding system on the occurrence of tetracycline-resistant Enterobacteriaceae (Ent-Tetr) in the liquid feed. 156 and 112 feed samples were collected between April and December 2015 in 13 case and 14 control farms, respectively. The 27 farms were randomly selected pig fattening farms located in different parts of Switzerland. The number of feed samples that contained Ent-Tetr as well as the number of Enterobacteriaceae resistant to tetracycline per sample was significantly higher in the case group than in the control group. The use of any of the 3 antimicrobial combinations turned out to be the main risk factor for the occurrence of Ent-Tetr in the liquid feed. Our results suggest that liquid feed containing antimicrobials is a reservoir of antimicrobial resistant bacteria in swine production.

Keywords: antimicrobial resistance, oral group therapy, tetracycline, Enterobacteriaceae, liquid feeding, fattening pigs

Zusammenfassung

Die orale Gruppentherapie ist eine immer noch verbreitete Managementmassnahme in der Schweineproduktion. Diese Arbeit untersuchte den Effekt der Applikation von 3 verschiedenen Formulierungen antibiotikahaltiger Arzneimittelvormischungen (1. Chlortetrazyklin, 2. Chlortetrazyklin + Sulfadimidin + Tylosin, 3. Sulfadimidin + Sulfathiazol + Trimethoprim) über die Flüssigfütterungsanlage auf das Vorkommen Tetrazyklin-resistenter Enterobacteraiaceae (Ent-Tetr) im Flüssigfutter. 156 bzw. 112 Futterproben wurden zwischen April und Dezember 2015 auf 13 Fall- bzw. 14 Kontrollbetrieben erhoben. Bei den 27 Betrieben handelte es sich um zufällig ausgewählte Schweinemastbetriebe aus verschiedenen Regionen der Schweiz. Die Anzahl Futterproben, die Tetrazyklin-resistente Enterobacteriaceae enthielten, sowie die Keimzahl Ent-Tetr pro Futterprobe waren signifikant höher in der Fall- als in der Kontrollgruppe. Der Einsatz der untersuchten Formulierungen von Arzneimittelvormischungen konnte als Hauptrisikofaktor für das Auftreten von Ent-Tetr im Flüssigfutter identifiziert werden. Die Ergebnisse legen nahe, dass antibiotikahaltiges Flüssigfutter ein Reservoir für Antibiotika-resistente Bakterien in der Schweineproduktion darstellt.

Schlüsselwörter: Antibiotikaresistenz, orale Gruppentherapie, Tetrazyklin, Enterobacteriaceae, Flüssigfütterung, Mastschweine

The effect of the administration of three different antimicrobial premix formulations via the liquid feeding system on the occurrence of Enterobacteriaceae resistant to tetracycline in the liquid feed for pigs

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Introduction

The annual amount of antimicrobials used for farm animals in Switzerland has continuously decreased from 71'316 kg in 2008 to 48'402 kg in 2014 (-32.1%) (ARCH-VET, 2014). In 2014, antimicrobial premixes (AMP) intended for adding to animal feed made up 60% of the total amount of antimicrobials used in farm animals. The amounts of antimicrobials presented in the ARCH-VET report (2014) are, however, not discriminated by animal species, age group and indication. A study conducted by Regula et al. (2009) on the prescription patterns in 8 veterinary practices in Switzerland found that 39% and 14% of the total amount of 1'590 kg of antimicrobials were prescribed for pigs and pigs or cattle, respectively. Under the assumption that this finding is representative for whole of Switzerland, the amount of AMP prescribed for pigs can be extrapolated to at least 11'326 kg or 23.4% of the total amount of antimicrobials used for farm animals in 2014. The treatment, prevention and control of bacterial diseases in pigs are often accomplished by oral antimicrobial group treatment, thus explaining the considerable proportion of antimicrobials used as feed additive in pigs. According to Müntener et al. (2013), piglets after weaning and fattening pigs after arrival in the fattening farm are the two age groups that are most frequently treated with antimicrobials. Riklin (2015) studied the antimicrobial use and the associated risk factors during the fattening period in 101 pig fattening farms in Switzerland and identified, based on the animal treatment index defined by Blaha et al. (2006), the prophylactic oral group treatment as the main indication (79%) for antimicrobial use in the fattening period. The treatment of various diseases by oral group treatment (18%) and individual parenteral treatment (3%) were distinctly less significant indications for using antimicrobials in fattening pigs. The most used AMPs for oral group treatments was a combination of sulfathiazole, sulfadimidine and trimethoprim, being followed by a triple combination containing chlortetracycline, sulfadimidine and tylosin and AMPs containing only chlortetracycline and colistin, respectively.
The antimicrobial oral group treatment can be performed by feeding a medicated feed mixed by the farmer himself, by adding the AMP into the mixing tank of a liquid feeding system, by mixing the feed and AMP directly in the trough (top dressing) or by feeding a medicated feed mixed by a feed mill. Liquid feeding is the main feeding system used in Swiss pig fattening farms. There are different construction types of liquid feeding systems, the easiest one consisting of a mixing tank, a feed scale under the mixing tank, a pump that pumps the liquid feed from the mixing tank trough the circuit pipeline to the drop lines which direct the liquid feed to the feed troughs. The feeding process is controlled by a computer that triggers the two unidirectional valves at the start and the end of the circuit pipeline and the dosing valves at the junctions connecting the circuit pipeline and the drop lines. In a liquid feeding system of this type, the liquid feed inside the circuit pipeline remains there between two feeding times, being diluted with water in some farms, and is

pumped back into the mixing tank during the mixing process. The drop lines are completely or partially free of feed between feeding times depending on the slope of the correspondent segments.
The liquid feeding systems are coated with a biofilm consisting, apart from water and bacteria, of different types of extracellular polymeric substances such as exopolysaccharides, proteins, nucleic acids and lipids (Flemming and Wingender, 2010). Antimicrobials are not only hindered by the biofilm matrix in their action against the microorganisms embedded in it, they may also induce the formation of biofilms dependant on the bacterial species and the antimicrobial concentration (Kaplan, 2011; Costa et al., 2012). The application of antimicrobials over a liquid feeding system therefore poses a risk of selecting antimicrobial resistant bacteria (ARB) in the biofilm by locally sub-inhibitory antimicrobial concentrations and the favourable conditions in the biofilm matrix for horizontal gene transfer (HGT) (Flemming and Wingender, 2010). The biofilm coating of a liquid feeding system may therefore ultimately be regarded as a reservoir of ARB, which can be detached and dispersed from the biofilm by mechanical forces or various biological processes at any time (Karatan and Watnick, 2009) and which are subsequently ingested by the pigs, thus adding further antimicrobial resistance (AMR) genes to the AMR gene pool already present in the pig gut. The administration of antimicrobials in pig fattening farms via “farm specific equipments”, which are mainly liquid feeding systems, accounted still for 20% of the chosen application method in a study conducted by Müntener et al. (2013). The commonly used antimicrobials in the AMPs sold in Switzerland for the use in prophylactic oral group treatment of fattening pigs are tetracycline and sulphonamide. The aim of this case-control-study was to quantitatively assess the effect of the administration of three different AMP formulations via the liquid feeding system on the occurrence of Enterobacteriaceae resistant to tetracycline (Ent-Tetr) in the liquid feed for pigs.

Material and Methods

Study design

For this case-control-study, 268 feed samples were collected between April and December 2015 at 27 pig fattening farms located in different areas of Switzerland. All farms used a computer-assisted liquid feeding system with water or whey as liquid phase. They all fed non-fermented liquid feed meaning that the conventional dry compound feed and the liquid were mixed immediately before feeding. The control group, encompassing 14 farms, was defined as farms that have not added any antimicrobials to the liquid feed for at least 2 years. The case group comprised 13 farms that administered AMP via the liquid feed in every fattening period of the last 2 years. Three different AMP formulations were used: chlortetracycline alone (3 farms, 23.1%), a combination of sulfadimidine, sulfathiazole and trimethoprim (7 farms, 53.8%) or a combination of chlortetracycline, sulfadimidine and tylosin (3 farms, 23.1%) (Tab. 1). The farms' individual routines applied for cleaning the liquid feeding system were not altered during the study. This approach allows for estimating the influence of the various management routines on the number of resistant isolates in the liquid feed. Table 2 summarises the cleaning interval, the cleaning procedure and the agents used for cleaning the circuit pipeline of the liquid feeding system.

Sample collection

Samples were collected at 4 time points in control and at 6 time points in case farms. In control farms, the second, third and fourth sample time were fixed on day 6, 12 and 78 after the collection of the first 2 samples. In case farms, the sampling started shortly after the entry of the pigs in the fattening unit but still before the medication. The remaining 5 sampling time points were scheduled on day 6, 12, 18, 36 and 76 after the start of the antimicrobial group treatment. The second sampling point was during medication in every case. At each time point, 2 samples were taken at 2 different locations, namely at the end of the circuit pipeline, which is situated right over the mixing tank, and at the end of the drop line that is the last one breaching off the circuit pipeline before its end. The feed samples were directly collected from either tube by holding a sterile container in the outflowing liquid feed without touching anything from the surroundings. As the liquid feed in all the investigated farms remains in the circuit pipeline between the feeding times, all the sampling was done at the morning feeding thus ensuring to collect samples from liquid feed that had interacted with the biofilm coating the inside of the tubes during the longest time period between two feeding times (10.5 – 15.75 hours).
Every farmer was interviewed about the use of antimicrobials, the construction and functioning of the liquid feeding system, the sanitary status of his farm, the type of feed and liquid phase, any potential acidification of the liquid feed and the different routines applied for cleaning the circuit pipeline, the drop lines, the mixing tank, the fodder silo and the whey tank, if there is any.

Microbiological analysis

All feed samples were kept cool during transport and were processed immediately upon arrival in the laboratory. The pH value of each sample was determined using a pH meter (Orion 525, Hügli, Abtwil). The quantitative assessment of the number of Enterobacteriaceae and Ent-Tetr was performed by means of 2 serial dilutions with a detection limit of 10 colony forming units/ml (cfu/ml) each. MacConkey agar (Oxoid, Hampshire, UK) and MacConkey agar supplemented with 8 mg/ml tetracycline (Sigma-

Aldrich, St. Louis, USA) were used for the detection of Enterobacteriaceae and Ent-Tetr, respectively. The MacConkey agar plates were incubated anaerobically during 24 h at 37° C. After the incubation, the colonies were counted and 1 colony of each morphological distinct resistant colony was subcultered using again a MacConkey agar supplemented with 8 mg/ml tetracycline and incubated anaerobically during 24 h at 37° C. This approach allows for the confirmation of resistance of the isolated colony.
In addition to the quantitative assessment of Ent-Tetr, the first 2 samples of every case farm and the last 2 samples of every control farm were enriched for Enterobacteriaceae using 10 ml of liquid feed and 90 ml of Enterobacteriaceae Enrichment (EE) broth (BD, Franklin Lakes, USA) and subcultured on MacConkey agar supplemented with 8 mg/ml tetracycline. This qualitative assessment allows for the detection of resistant Enterobacteriaceae in case the number of the same is less than the detection limit of 10 cfu/ml. Furthermore, this approach allows for determining whether or not there were Ent-Tetr even before the antimicrobial group treatment.

Statistical analysis

Data were analysed using the commercial statistical software Stata (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). The number of Enterobacteriaceae and Enterobacteriaceae resistant to tetracycline represented the dependent variables (observations) and were initially analysed by means of descriptive statistics. The independent variables were defined as group affiliation (case/control group), sample location (drop line = sampling site 1/circuit pipeline = sampling site 2), sample time (case group: $t_1,\ldots,t_6$; control group $t_1,\ldots,t_4$), type of AMP (AMP containing chlortetracycline/AMP containing only chlortetracycline), type of liquid phase (water/whey), cleaning of the circuit pipeline after the antimicrobial group treatment (yes/no), acidification of the liquid feed (yes/no). Each farm was assigned either to the case group or the control group. Therefore there was no clustering of farms when comparing the different sampling sites. Test for normality distribution was performed using the Shapiro–Wilk test with the significance level of 95% (p-value ≤ 5%). As all data were not normally distributed and no transformation showed sufficient Gaussian distribution, the initial comparison between independent variables was performed using non-parametric tests, i.e. the Wilcoxon rank-sum test (Mann-Whitney U test) in case of two independent groups and the Kruskal-Wallis test in case of more than two groups. A general linear model was calculated (STATA command <by varx1, sort : xtmixed vary varx2 vart || vart>; varx1 = sample location, varx2 = group affiliation, vary = number of Ent-Tetr per sample, vart = time). Multiple non-parametric comparisons between several groups, e.g. observations at different sample times, were done with Dunn's test of multiple comparisons using rank sums with Bonferroni adjustment. With all three tests, the significance level was chosen to be 95% (p-value ≤ 5%). In order to identify risk factors for the occurrence of Ent-Tetr in the liquid feed, the results of the univariate logistic regression models were used to define a full logistic regression model with random effects (STATA command <xtset vart>, <xtlogit vary varx vart>; varx = list of independent variables, vary = tetracycline resistance (dichotomous), vart = time). The univariate analysis was performed with 6 time points in case farms and with 4 time points in control farms. The multivariate analysis, however, was based only on the 4 time points which existed in both groups. The cut-off value for the dichotomous dependent variable tetracycline resistance corresponded with the detection limit of 10 cfu/ml for Ent-Tetr. The selection of the final risk factors was done by backward elimination so that the p-value of every risk factor was less than or equal to 0.05.

Results

Enterobacteriaceae could be isolated from 119 (76.3%) samples of the case group and from 63 (56.3%) samples of the control group (Tab. 3). In the case group, 68 (87.2%) samples collected at sampling site 1 and 51 samples (65.4%) collected at sampling site 2 contained Enterobacteriaceae. The corresponding values for the control group were 35 (62.5%) samples at sampling site 1 and 28 (50%) samples at sampling site 2, respectively. Tetracycline-resistant Enterobacteriaceae could be isolated from 104 (66.7%) samples of the case group and 7 (6.3%) samples of the control group. In the case group, 61 (78.2%) samples collected at sampling site 1 and 43 (55.1%) samples collected at sampling site 2 contained Ent-Tetr. The corresponding values for the control group were 7 (12.5%) at sampling site 1 and 0 (0%) at sampling site 2, respectively.
There was no case farm where the number of Ent-Tetr in all 6 samples at sampling site 1 was below the detection limit of 10 cfu/ml (Tab. 4). However, there were 2 case farms where all 6 samples collected at sampling site 2 were below the detection limit of 10 cfu/ml for Ent-Tetr. Nonetheless, Ent-Tetr could be isolated in the samples of these two latter case farms taken at the first sample time and at sampling site 2 after an enrichment for Enterobacteriaceae had been performed. In summary, Ent-Tetr could be isolated in all case farms at both sampling sites by either using a quantitative or qualitative approach. Furthermore, all samples (100%) at sampling site 1 and all but 1 (92.3%, case farm 7) samples at sampling site 2, collected before the antimicrobial group treatment, contained Enterobacteriaceae resistant to tetracycline.

There were 9 (64.3%) and 14 (100%) control farms, respectively, where the number of Ent-Tetr was below the detection limit of 10 cfu/ml in all 4 samples at sampling sites 1 and 2, respectively (Tab. 4). The enrichment for Enterobacteriaceae revealed that the control farms 3 and 4 (14.3%) were free of Ent-Tetr at both sampling sites, whereas control farms 5, 6, 8, 9, 10 and 12 (42.9%) were free only at sampling site 2. The control farms 1, 2, 7, 11, 12 and 14 (42.9%) were positive at both sampling sites in the qualitative approach. In summary, there were 2 (14.3%) and 6 (42.9%) control farms, which were negative for Ent-Tetr at sampling sites 1 + 2 or sampling site 2 only, respectively, using either a qualitative or a quantitative approach.

The number of Ent-Tetr was higher in the case group than in the control group at sampling sites 1 + 2 ($p < 0.0001$), at sampling site 1 ($p < 0.0001$) and at sampling site 2 ($p < 0.0001$) (Tab. 3, Fig. 1). The number of Ent-Tetr differed significantly between the sample times in the case group at sampling sites 1 + 2 ($p = 0.0401$) (Fig. 2) and at sampling site 1 ($p = 0.0242$), but not in the control group ($p = 0.6860$). The relative frequency of the number of Ent-Tetr was higher in the case group than in the control group at sampling sites 1 + 2 ($p < 0.0001$) (Fig. 3 and 4), at sampling site 1 ($p < 0.0001$) and at sampling site 2 ($p < 0.0001$). The relative frequency was higher in farms that used water as liquid phase than in farms that mixed feed with whey at sampling sites 1 + 2 ($p = 0.0295$). The relative frequency did not differ between farms that cleaned their circuit pipeline after the group treatment and those that did not ($p = 0.2371$).

The univariate logistic regression analysis identified the *use of antimicrobials* (OR = 30.0, CI 13.0 – 69.1, $p < 0.001$), *sampling site 1* (OR = 2.2, CI 1.3 – 3.6, $p = 0.002$), the *use of water* (OR = 1.9, CI 1.1 – 3.4, $p = 0.021$) and the *lack of acidification* (OR = 4.1, CI 2.2 – 7.6, $p < 0.001$) as risk factors for detecting Ent-Tetr in the sample with colony counts higher than 10 cfu/ml (Tab. 5). In the final multivariate logistic regression model, only the risk factors *use of antimicrobials* (OR = 58.9, CI 20.9 – 166.5, $p < 0.001$), *sampling site 1*

(OR = 5.4, CI 2.2 – 13.5, $p < 0.001$) and *lack of acidification* (OR = 4.9, CI 1.8 – 12.9, $p = 0.001$) were left. The logistic regression analysis was also performed using only data from the case group. In the univariate logistic regression model, *sampling site 1* (OR = 2.9, CI 1.5 – 5.9, $p = 0.003$), *lack of acidification* (OR = 5.6, CI 2.5 – 12.3, $p < 0.001$) and *use of an AMP with only chlortetracycline* (OR = 3.1, CI 1.2 – 8.0, $p = 0.019$) could be identified as risk factors. In the final multivariate logistic regression model, only the risk factors *sampling site 1* (OR = 4.3, CI 1.6 – 11.7, $p = 0.004$) and *lack of acidification* (OR = 6.3, CI 2.2 – 18.3, $p = 0.001$) were left.

The duration of the antimicrobial group treatment varied between 6 and 10 days (Tab. 1). If the criteria for a correct dosage (+/- 10%) described by Regula et al. (2009) is applied, then 6 (46.2 %) farms applied a correct dosage, whereas 4 (30.8 %) and 3 (23.1 %) farms under- or overdosed, respectively. The daily dosage was applied in 5 (38.5 %) farms at 1 feeding in the morning, in 6 (46.2 %) farms at 2 feedings in the morning and afternoon or evening and in 2 (15.4 %) farms at 3 feedings. All case farms reduced the amount of liquid feed by a factor of 50 to 60 % by the time the pigs entered their farm and subsequently increased it to 100 % of the daily energy demand within a time span of eight to ten days. The daily applied amount of AMP stayed the same during the whole treatment period, which is the reason why the concentration of the antimicrobials in the liquid feed decreased from the start to the end of the antimicrobial group treatment (Tab. 1). Only 1 (7.7 %) and 4 (30.8 %) case farms cleaned their drop lines and circuit pipeline, respectively, after the antimicrobial medication. 12 (92.3 %) and 13 (100 %) case farms cleaned their circuit pipeline and drop lines, respectively, after the end of the fattening period. The cleaning of the drop lines was in all cases performed by means of cold or warm water and a high-pressure hose, which was inserted into the drop lines from their free end. There was 1 case farm that flushed its circuit pipeline only with water after the end of the fattening period and out of the 12 case farms that cleaned their circuit pipeline with some sort of agent, there was 1 farm that used a silage additive instead of a proper cleaning agent. In the control group, there were 4 (28.6 %) and 6 (42.9 %) farms that have not cleaned their circuit pipelines and drop lines, respectively, for several years.

Discussion

To the authors' knowledge, this is the first report on the abundance of Ent-Tetr in the liquid feed for pigs as a function of the short-term use of in-feed antimicrobials. Our results suggest that feeding therapeutic doses of any of the three most frequently used antimicrobial combinations in Switzerland (Riklin, 2015) is the main risk factor for the presence of Ent-Tetr in the liquid feed. Other risk factors identified in this study encompass the lack of acidification of the liquid feed and the sampling of fluid feed from the end of the drop line.

The selective pressure that is exerted by orally administered antimicrobials on the bacterial population in the liquid feed is reflected by the markedly higher proportion of feed samples containing Enterobacteriaceae resistant to tetracycline and by the higher number of cfu/ml of Ent-Tetr in the case group than in the control group. Antimicrobial resistant bacteria arise by mutations or by the acquisition of antimicrobial resistance determinants by HGT (Andersson and Hughes, 2011; Huddleston, 2014; van Schaik, 2015). It is well documented and widely accepted that the use of a given antimicrobial is the driving force behind the selection of bacteria resistant to the applied antimicrobial agent (Davies and Davies, 2010; Forslund et al., 2013; Modi et al. 2014). Interestingly, resistance to an antimicrobial can also be selected by the use of a structurally related (cross-selection) or unrelated (co-selection) antimicrobial (Guardabassi and Kruse, 2008). The latter is the most probable explanation for our findings that there was no significant difference in the odds for resistance to tetracycline depending on whether or not the used AMP contained chlortetracycline. Resistance genes for trimethoprim-sulfametoxazole and tetracycline are reported to be often located on the same conjugative plasmid (Geser et al., 2012). Gibbons et al. (2015) identified the use of antimicrobial combinations containing sulphonamide and trimethoprim as a risk factor for the occurrence of *E. coli* resistant to tetracycline in the faeces of pigs.

The application of antimicrobials via the liquid feeding system seems to be further associated with a quantitative shift in the population of Enterobacteriaceae from a predominantly tetracycline-susceptible towards a tetracycline-resistant population, which is reflected by the significantly higher relative frequency of Ent-Tetr in the liquid feed samples from case farms compared to those from control farms. These findings are in accordance with results from previous studies that link the use of antimicrobials with an increased frequency of ARB (Aminov, 2009; Andersson and Hughes, 2011).

There are several beneficial effects of the addition of organic acids to pig feed. The main effect of dietary organic acids lies in the reduction of the pH in the stomach by lowering the acid buffering capacity of the feed, thus reducing the amount of commensals as well as pathogenic bacteria, e.g. *Salmonella spp.*, in the swine gut (Suiryanrayna and Ramana, 2015). While there was no significant difference in the pH between farms that used organic acids and those that did not (Tab. 4), the total number of Ent-Tetr as well as the number of samples containing Enterobacteriaceae resistant to tetracycline was significantly lower in samples from farms that added acids to the liquid feed (data not shown). Our data suggest that the use of organic acids is a protective factor for tetracycline resistance. As the case farms that applied acids were not the same as those that cleaned their circuit pipelines, the calculation of the corresponding odds ratios was not reciprocally influenced.

All findings suggest a long-lasting effect of a repeated, short-term use of antimicrobials via the liquid feeding system on the abundance of Ent-Tetr in the liquid feed. However, the cleaning of the circuit pipeline after the medication in 4 case farms by means of a disinfectant was marked by a sharp decrease in the number of cfu/ml of Ent-Tetr at both sampling sites. As the sampling process was not continued in the following fattening period, it was not possible to determine whether the level of resistance in these 4 case farms

remained at a low level or whether the reservoir of resistance in the liquid feeding might have expanded between the two fattening periods, i.e. when the liquid feeding systems were not in operation.

The median of the number of cfu/ml of Ent-Tetr was quite low at the end of the drop line (155 cfu/ml). However, it has to be considered that every fattening pig is fed, depending on its body weight, the energy content of the dry feed and fluid as well as the water to fluid ratio of the mixed liquid feed, a daily amount of 4 - 11 litre of liquid feed. Thus, it can be estimated, based on the quantitative assessment of tetracycline resistance in this study, that a fattening pig ingests approximately $6.2*10^5$ - $1.7*10^6$ Ent-Tetr every day. Looft et al. (2012) conducted a case-control study to investigate the effect of administering an in-feed antimicrobial combination (chlortetracycline, sulfamethazine, penicillin) over 14 days on the swine intestinal microbiome. They reported a significant shift in the composition of the bacterial community in the gut, e.g. a distinct proliferation of *E. coli*, and an increase in abundance and in diversity of AMR genes as a result of the antimicrobial treatment.

This study has some limitations. Resistance to tetracycline was not determined in the individual components of the analysed liquid feed (water, whey, dry feed). It was therefore not possible to assess the proportion of ARB introduced from outside of the liquid feeding system. Cleaning routines and agents, as well as the use of organic acids were, purposely, not standardised. The authors' intention was to assess the level of resistance to tetracycline in an average Swiss pig fattening farm and the chosen approach allowed to level out the substantial differences in the individual management routines among different farms. On the other hand, the robustness of the estimated risk factors could be impaired by this approach as it cannot be ruled out that not identified cofounders might have substantially influenced the results.

We could identify medicated liquid feed as a potential source of ARB. Prophylactic antimicrobial group treatment in fattening pigs aims at preventing bacterial infections in periods of high risks such as after weaning, transport and mixing of animals from different farms (Schwarz et al., 2001; McEwen and Fedorka-Cray, 2002; Callens et al., 2012; Trauffler et al., 2014). The most frequently used antimicrobial agents used prophylactically in Swiss pig fattening farms (Riklin, 2015) are broad-spectrum antimicrobials and are classified as critically important (macrolides) or highly important (tetracyclines, sulphonamides and trimethoprim) for human medicine by the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) (Anonymous, 2012). As broad-spectrum antimicrobials are known to promote AMR (Barbosa and Levy, 2000), the guidelines for prudent use of veterinary drugs defined by the Swiss Veterinary Society (GST) (Brügger, 2010) advise veterinarians to use an antimicrobial with a spectrum as narrow as possible. It lies in the nature of prophylaxis that there is no specifically targeted microorganism and therefore complying with the guidelines is inherently not possible when using antimicrobials prophylactically. The national Strategy for Antibiotic Resistance (StAR) defines various measures in order to obtain its main goal, which is the preservation of efficacy of antimicrobial agents (Anonymous, 2015). One of the measures includes the revision of the above-mentioned guidelines and to declare them binding. It is further planned to introduce legally binding prescription limitations in veterinary medicine based on the categorisation by AGISAR (Anonymous, 2012) and to restrict the prophylactic antimicrobial use. The start of implementation of StAR was scheduled for 2016 and farmers as well as veterinarians are well advised to prepare for the far-reaching changes in pig production. However, the study by Riklin (2015) revealed that the prophylactic oral group treatment in Swiss fattening pigs was not associated with a lower overall mortality rate, a lower individual treatment frequency or an increased productivity. Hence, a profitable production of healthy swine seems to be realistic even under stricter legal regulations.

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Tables, figures and annotations

Table 1: Antimicrobial group treatment regimes applied by the 13 case farms and the concentrations of the antimicrobials in the liquid feed at the start and the end of the treatment period. [1] Zoetis Schweiz GmbH, [2] Ufamed AG, [3] Vital AG.

Farm	AMP	Treatment days	Prescribed daily dose	Administered daily dose	Medicated feedings per day	Antimicrobial 1			Antimicrobial 2			Antimicrobial 3		
						Name	mg/kg feed		Name	mg/kg feed		Name	mg/kg feed	
			g AMP/100 kg LW	g AMP/100 kg LW			Start	End		Start	End		Start	End
Case 1	Vital CST-222 L [3]	7	40	27.2	3	Chlortetracycline	50	31	Sulfadimidine	92	57	Tylosin	9	5
Case 2	UFA 902 Duo [2]	6	50	30.3	1	Sulfadimidine	114	58	Sulfathiazole	114	58	Trimethoprim	46	23
Case 3	CAS 45 K [2]	7	30	36.6	3	Chlortetracycline	81	44	Sulfadimidine	131	70	Tylosin	13	7
Case 4	UFA 902 Duo [2]	10	50	50.0	2	Sulfadimidine	83	56	Sulfathiazole	83	56	Trimethoprim	33	23
Case 5	Aurofac® 100 [1]	8	30	35.7	1	Chlortetracycline	264	207	-	-	-	-	-	-
Case 6	Vital TSS 96 [3]	8	50	50.0	1	Sulfadimidine	173	84	Sulfathiazole	173	84	Trimethoprim	69	33
Case 7	Vital TSS 96 [3]	8	50	56.3	2	Sulfadimidine	116	72	Sulfathiazole	116	72	Trimethoprim	46	29
Case 8	Aurofac® 100 [1]	10	20	19.9	1	Chlortetracycline	177	86	-	-	-	-	-	-
Case 9	Aurofac® 100 [1]	10	20	20.7	2	Chlortetracycline	102	47	-	-	-	-	-	-
Case 10	UFA 902 Duo [2]	10	50	51.9	2	Sulfadimidine	89	45	Sulfathiazole	89	45	Trimethoprim	36	18
Case 11	UFA 902 Duo [2]	10	50	43.9	2	Sulfadimidine	66	32	Sulfathiazole	66	32	Trimethoprim	26	13
Case 12	Vital CST-222 L [3]	10	30	29.0	1	Chlortetracycline	98	81	Sulfadimidine	181	150	Tylosin	17	14
Case 13	UFA 902 Duo [2]	10	50	43.6	2	Sulfadimidine	36	26	Sulfathiazole	36	26	Trimethoprim	14	10

Table 2: Cleaning interval, cleaning agents and procedure (concentration, exposure time) of the 13 case and 14 control farms, * silage additive.

Farm	Cleaning interval	Cleaning agent	Procedure
Case 1	After end of fattening period	Vital: Venno-Vet 1 Super	1%, 15 minutes
Case 2	Every week and after end of fattening period	Halag: Halades 01	0.5%, 12 hours
Case 3	After end of fattening period	Halag: Stallcip 666	3%, 0.5 - 1 hour
Case 4	After end of fattening period	Amstutz: MILK-KLENE AD F	2%, 24 hours
Case 5	Every 10 days and after end of fattening period	Selko: Anti-Entero plus	3%, 10.5 hours
Case 6	After end of fattening period	water	rinsing
Case 7	After treatment and end of fattening period	1. 3 - 4 kg barley grains 2. Caustic soda	1. Flushing the tubes with water containg barley 2. 1%, 5 minutes
Case 8	After end of fattening period	Amstutz: MILK-KLENE AD F	2%, 24 - 48 hours
Case 9	After end of fattening period	Halag: Stallcip 666	2%, 20 - 25 minutes
Case 10	After end of fattening period	1. GEA Farm Technologies: CircoSuper AFM 2. H_2O_2	1. 3.7%, 30 minutes 2. 0.5%, 4 hours
Case 11	After end of fattening period	Halag: Stallcip 666	2%, 30 minutes
Case 12	After end of fattening period	1. Halag: Halades 01 2. Amstutz: MILK-KLENE AD F	1. 3.5%, 48 hours 2. 3.5%, 48 hours
Case 13	1. Every day 2. After treatment and end of fattening period	1. Halag: Halades 01 2. Halag: Stallcip 666	1. 0.5%, 12 hours 2. 2%, 24 hours
Control 1	Twice per year	Vital: Venno-Vet 1 Super	1%, 30 minutes
Control 2	Once per year	Halag: Pasteurreiniger 405	2%, 30 minutes
Control 3	No cleaning since 1987		
Control 4	Every 4 - 6 months	Arkema: H_2O_2 (35%)	14%, 1 hour
Control 5	No cleaning for at least 3 years		
Control 6	After end of fattening period	Caustic soda	5.8%, 12 hours
Control 7	Every 3 months	GEA Farm Technologies: CircoPower AFM	0.3%, 5 minutes
Control 8	Once per week	Alltech: Sil-All Fireguard *	0.8%, 12 hours
Control 9	No cleaning since construction (15 years ago)		
Control 10	Every 2 weeks	Halag: Halades 01	0.5 - 1%, 11 hours
Control 11	Every 3 - 4 months	Halag: Stallcip 666	2%, 30 - 40 minutes
Control 12	Every 3 months	Selko: Anti-Entero plus	3 - 6 ‰, 12 hours, on 4 consecutive days
Control 13	No cleaning for at least 5 years		
Control 14	Every 3 months	Selko: Anti-Hefen	0.5%, 10 hours

Table 3: Number of samples per sampling site, number of samples with at least 10 cfu/ml of Enterobacteriaceae or Enterobacteriaceae resistant to tetracycline (positive sample), mean, median and maximum of the observations, listed by group affiliation.

		Enterobacteriaceae			Tetracycline-resistant Enterobacteriaceae		
		Sampling site 1 + 2	Sampling site 1	Sampling site 2	Sampling site 1 + 2	Sampling site 1	Sampling site 2
Case group	Samples	156	78	78	156	78	78
	Positive samples (%)	119 (76.3)	68 (87.2)	51 (65.4)	104 (66.7)	61 (78.2)	43 (55.1)
	Mean [cfu/ml]	52'403.1	56'210.6	48'595.6	16’099.9	23’329.9	8’869.9
	Median [cfu/ml]	160	325	60	75	155	10
	Maximum [cfu/ml]	1'620'000	1'620'000	1'033'000	426’000	426’000	221’000
Control group	Samples	112	56	56	112	56	56
	Positive samples (%)	63 (56.3)	35 (62.5)	28 (50.0)	7 (6.3)	7 (12.5)	0 (0)
	Mean [cfu/ml]	30'242.6	18'936.2	41'549.1	11.2	17.4	5
	Median [cfu/ml]	15	75	7.5	5	5	5
	Maximum [cfu/ml]	2'320'000	910'000	2'320'000	300	300	5

Table 4: Samples with at least 10 cfu/ml of Ent-Tetr (positive samples, grey shaded), listed by group affiliation, and number of positive samples at different sample times, listed by group affiliation. Clean = Cleaning of the circuit pipeline after the medication, Acid = Addition of organic acids to the liquid feed.

Farm	Sample	Clean	Acid	Drop line [cfu/ml]	Circuit pipeline [cfu/ml]
Case 1	1		YES	19'500	
	2			50	60
	3			740	700
	4				
	5			20	10
	6			100	490
Case 2	1	YES		5'560	9'500
	2				
	3				
	4				
	5			70	
	6			190	90
Case 3	1			230	0
	2			35'800	58'000
	3			6'300	1'100
	4			11'000	2'000
	5			9'800	10
	6			7'500	890
Case 4	1		YES	5'000	
	2				
	3				
	4				
	5				
	6				
Case 5	1	YES		11'000	30
	2			11'900	
	3			150	240
	4			30	10
	5			20	30
	6			270	1'300
Case 6	1			37'400	29'100
	2			46'200	2'400
	3			200	300
	4			53'700	32'500
	5			213'000	100'400
	6			5'700	6'200
Case 7	1	YES		940	
	2			30	
	3			10	20
	4			20	
	5			100	
	6			10	
Case 8	1			29'700	18'200
	2			250'000	87'400
	3			426'000	730
	4			3'200	170
	5			4'100	100
	6			160	1'800
Case 9	1			36'700	100'800
	2			280	
	3			340	330
	4			20	20
	5				
	6				
Case 10	1			410'000	10
	2			6'500	1'300
	3			5'200	3'800
	4			2'200	740
	5			1'300	1'200
	6			50	90
Case 11	1			157'000	221'000
	2			310	
	3			30	
	4				
	5			520	1'270
	6			80	30
Case 12	1		YES		
	2				
	3			30	
	4			110	
	5				
	6			80	
Case 13	1	YES		3'100	7'300
	2			80	10
	3				
	4			10	
	5				
	6			10	
Positive samples				61 (78.2 %)	43 (55.1 %)
Number of samples				78	78

Farm	Sample	Acid	Drop line [cfu/ml]	Circuit pipeline [cfu/ml]
Control 1	1	YES		
	2			
	3			
	4			
Control 2	1	YES		
	2			
	3			
	4		10	
Control 3	1			
	2			
	3			
	4			
Control 4	1	YES		
	2			
	3			
	4			
Control 5	1	YES		
	2			
	3			
	4			
Control 6	1	YES		
	2			
	3		70	
	4			
Control 7	1			
	2			
	3			
	4		20	
Control 8	1			
	2		300	
	3			
	4		30	
Control 9	1			
	2			
	3			
	4			
Control 10	1			
	2			
	3			
	4			
Control 11	1			
	2			
	3			
	4			
Control 12	1			
	2			
	3			
	4			
Control 13	1			
	2			
	3			
	4			
Control 14	1		270	
	2		30	
	3			
	4			
Positive samples			7 (12.5 %)	0 (0 %)
Number of samples			56	56

Case group ($n(t_i) = 13$; $i = 1,...,6$)		
Positive samples, t1	12 (92.3 %)	8 (61.5 %)
Positive samples, t2	10 (76.9 %)	6 (46.2 %)
Positive samples, t3	10 (76.9 %)	8 (61.5 %)
Positive samples, t4	9 (69.2 %)	6 (46.2 %)
Positive samples, t5	9 (69.2 %)	7 (53.8 %)
Positive samples, t6	11 (84.6 %)	8 (61.5 %)

Control group ($n(t_i) = 14$; $i = 1,...,4$)		
Positive samples, t1	1 (7.1 %)	0 (0 %)
Positive samples, t2	2 (14.3 %)	0 (0 %)
Positive samples, t3	1 (7.1 %)	0 (0 %)
Positive samples, t4	3 (21.4 %)	0 (0 %)

Table 5: Univariate and multivariate logistic regression analysis. The upper part of the table shows the results based on the data from the 27 case and control farms, whereas the results in the lower part of the table were calculated based on the data from the 13 case farms. The full dataset of the case group (6 time points) was used to calculate the sample numbers in the 2nd and 3rd column. CI = confidence interval.

Potential risk factor	Samples with resistant isolates (n = 111)	Samples without resistant isolates (n = 157)	Univariate analysis		Multivariate analysis	
			Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value
Use of antimicrobials	104	52	30.0 (13.0 – 69.1)	< 0.001	58.9 (20.9 – 166.5)	< 0.001
Sampling site 1 (drop line)	68	66	2.2 (1.3 – 3.6)	0.002	5.4 (2.2 – 13.5)	< 0.001
Use of water	88	104	1.9 (1.1 – 3.4)	0.021	-	-
Lack of acidification	96	96	4.1 (2.2 – 7.6)	< 0.001	4.9 (1.8 – 12.9)	0.001

Potential risk factor	Samples with resistant isolates (n = 104)	Samples without resistant isolates (n = 52)	Univariate analysis		Multivariate analysis	
			Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value
Sampling site 1 (drop line)	61	17	2.9 (1.5 – 5.9)	0.003	4.3 (1.6 – 11.7)	0.004
Use of water	83	37	1.6 (0.7 – 3.5)	0.229	-	-
No cleaning after treatment	75	33	1.5 (0.7 – 3.0)	0.271	-	-
Lack of acidification	91	29	5.6 (2.5 – 12.3)	< 0.001	6.3 (2.2 – 18.3)	0.001
Use of an AMP with chlortetracycline	53	19	1.8 (0.9 – 3.6)	0.090	-	-
Use of an AMP without chlortetracycline	51	33	0.6 (0.3 – 1.1)	0.090	-	-
Use of an AMP with only chlortetracycline	30	6	3.1 (1.2 – 8.0)	0.019	-	-

Annotations to figures

Figure 1: Boxplot of the number of cfu/ml of Ent-Tetr in the liquid feed of 13 case and 14 control farms, collected at the end of the last drop line (sampling site 1) or at the end of the circuit pipeline (sampling site 2).

Figure 2: Boxplot of the number of cfu/ml of Ent-Tetr in the liquid feed of 13 case farms at 6 sample times and at sampling sites 1 + 2.

Figure 3: Boxplot of the relative frequency of the number of cfu/ml of Ent-Tetr in the liquid feed of 13 case farms at 6 sample times and at sampling sites 1 + 2.

Figure 4: Boxplot of the relative frequency of the number of cfu/ml of Ent-Tetr in the liquid feed of 14 control farms at 4 sample times and at sampling sites 1 + 2.

Figure 1:

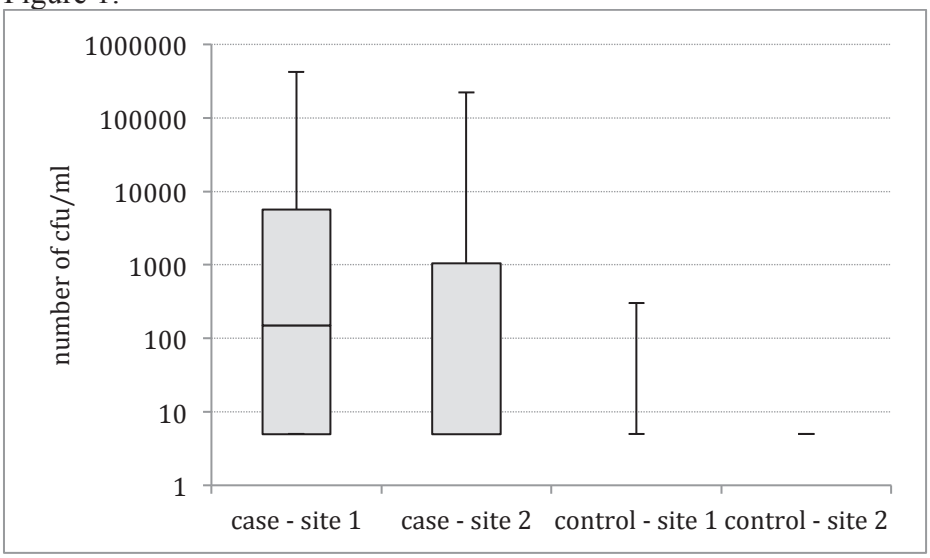


Figure 2:

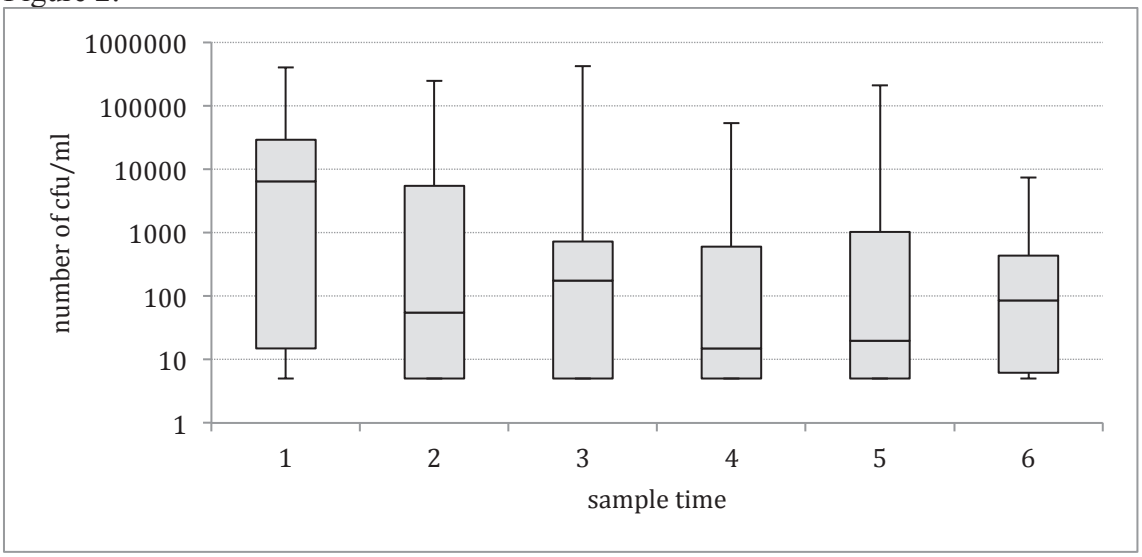


Figure 3:

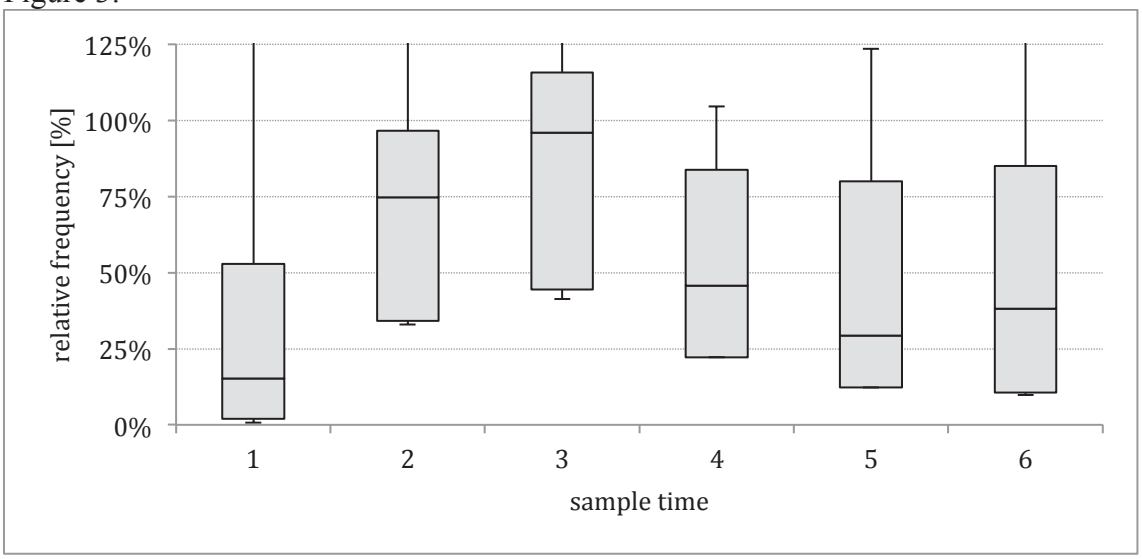
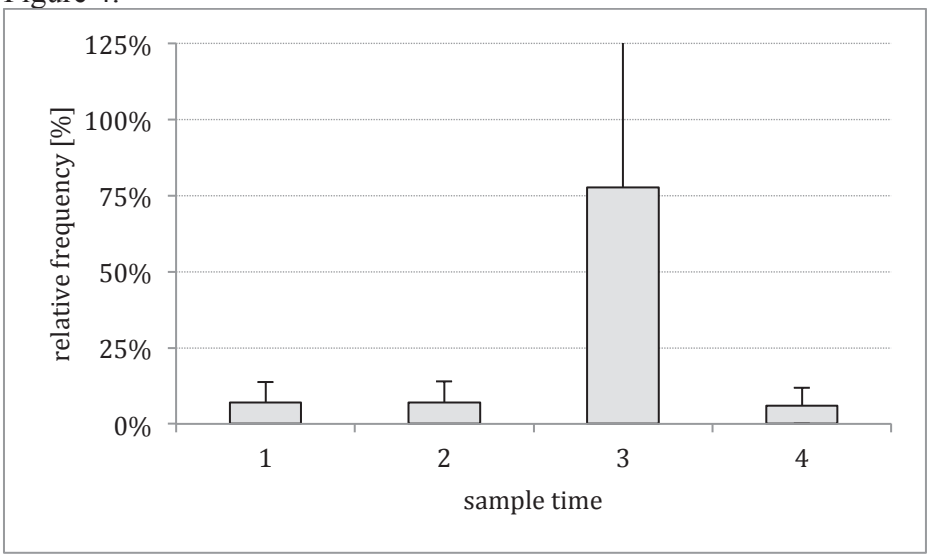


Figure 4:



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